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The *pkI* Gene Encoding Pyruvate Kinase I Links to the *luxZ* Gene Which Enhances Bioluminescence of the *lux* Operon from *Photobacterium leiognathi*

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Partial 3′-end nucleotide sequence of the *pkI* gene (GenBank accession No. AF019143) from *Photobacterium leiognathi* ATCC 25521 has been determined, and the encoded pyruvate kinase I is deduced. Pyruvate kinase I is the key en-zyme of glycolysis, which converts phosphoenol pyruvate to pyruvate. Align-ment and comparison of pyruvate kinase Is from *P. leiognathi, E. coli* and *Sal-monella typhimurium* show that they are homologous. Nucleotide sequence re-veals that the *pkI* gene is linked to the *luxZ* gene that enhances bioluminescen-ce of the *lux* operon from *P. leiognathi.* The gene order of the *pkI* and *luxZ* genes is -*pk1-ter* → -*R&R"-luxZ-ter"*→, whereas *ter* is transcriptional termina-tor for the *pkI* and related genes, and *R&R″* is the regulatory region and *ter″* is transcriptional terminator for the *luxZ* gene. It clearly elicits that the *pkI* gene and *luxZ* gene are divided to two operons. Functional analysis confirms that the potential hairpin loop ΩT is the transcriptional terminator for the *pkI* and related genes. It infers that the *pkI* and related genes are simply linked to the *luxZ* gene in *P. leiognathi* genome. © 1997 Academic Press

Luminous bacteria emit blue-green or blue bioluminescence (λ_{max} 490-505 nm or 470 nm) in nature. As known, luciferase is the enzyme responsible for the bioluminescence reaction. Overall reaction catalyzed by luciferase is:

$$RCHO + FMNH_2 + O_2 \rightarrow RCOOH + FMN + H_2O + h\nu$$

As depicted, the genes responsible for bioluminescence reaction form the *lux* oper-on, which included the *luxC-DABE;* whereas the *luxA* and *luxB* genes encode the α and β subunits of luciferase, the *luxC, luxD* and *luxE* encode the enzymes formed fatty acid reductase complex that is responsible to convert fatty acid to aldehyde as substrate for the reaction; there the *luxC* gene encodes fatty acid reductase, the *luxD* gene encodes acyltransferase and the *luxE* gene encodes acyl-protein synthetase, all of the three enzyme form fatty acid reductase complex for bioluminescence reaction (1-3). It was already known that the gene order of the *lux* and *lum* operons from *P. leiognathi* PL741 is ←*putA-R&R'-←ter-lumQ-lumP-R&R-luxC-luxD-luxA-luxB-luxN-luxE*→; there the *luxN* gene encodes non-fluorescent flavoprotein (2-15). In addition, the *luxZ* gene that enhances bioluminescence of the *lux* operon from *P. leiognathi* in *E. coli* was cloned by *in trans* complementation bioluminoassays *in vivo* (16). To investigate the genes closely linked to the *luxZ* gene might provide infor-mation to understand the relationship of these genes.

MATERIALS AND METHODS

Bacterial strains, plasmids, and plasmid construction. Photobacterium leiognathi ATCC 25521 was used for genome library construction by *in trans* complementation biolu-minoassays *in vivo. E. coli* JM103y is a phage P1-free derivative of JM103 and which was used as host for phage M13 derivatives and cloning works. Plasmid pL741, which carried the *lum*, the *lux* operons and the related genes, was cloned from *P. leiognathi* PL741 genomic DNA (7, 10-15). Terminator-proving vector pYFL1 was used for functional ana-lysis of the potential hairpin loop by bioluminoassays *in vivo;* whereas the *luxA* and *luxB* genes of luciferase from *V. harveyi* B392 (ATCC 33843) were used as reporter genes in pYFL1.

Nucleotide sequence and amino acid sequence analyses. Nucleotide sequence was obtained by means of the dideoxy chain termination method (17) and the modified double strand DNA sequencing method. pUC18/19 and M13mp18/mp19 RF-DNA were used as DNA sequencing vectors. α-P^{32}-dATP was used for DNA labeled. Nucleotide sequence and amino acid sequence data were analyzed with a PC/GENE Program (6.85 version) and GCG Program.

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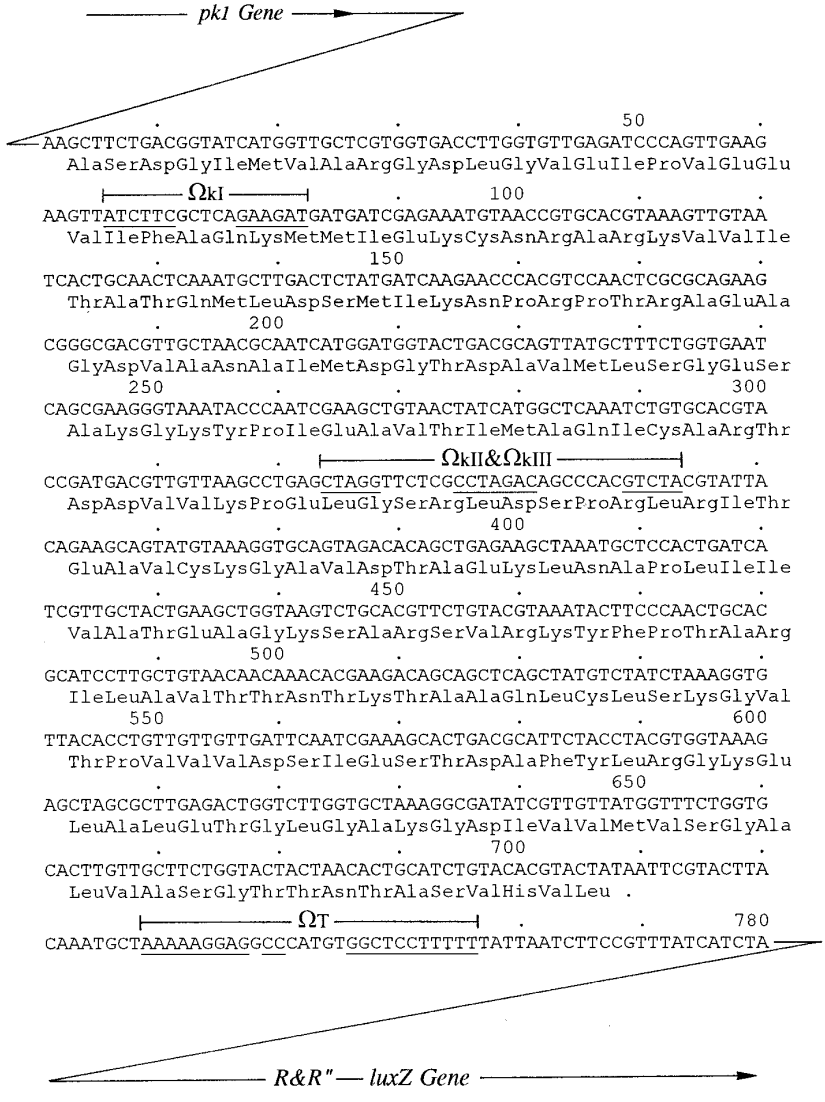

FIG. 1. Nucleotide sequence of the *pkI* gene and the encoded pyruvate kinase I from *P. leiognathi* ATCC 25521. Partial 3′-end nucleotide sequence and open reading frame (ORF from 1st to 710th bp) of the *pkI* gene is shown; the potential loops ΩkI, ΩkII, ΩkIII and transcriptional terminator ΩT resided in or behind the *pkI* gene are indicated. The nucleotide sequence has been deposited at GenBank data-base under accession No. AF019143.

Standard assays of luciferase activity and bioluminoassays in vivo. Luciferase activity in the cells was determined according to the standard assay described by Hastings *et al.* (18). Bioluminoassays *in vivo,* n-decanal (0.1%, v/v in ethanol) (1-5 μl) was added to cell culture (100 μl) to do the assay. Luminometer Bio-Orbit 1251 was used to calibrate the light emission, and bioluminescence was shown as V/ml (volts/ml).

Media and growth conditions. Luria-Bertani (LB) medium was used for E. coli grow-th. Normal growth condition for *E. coli* was 37°C with agitation. Various growth condi-tions are required for optimal gene expression. Cells taken from permanent stock were used to start overnight cultures in LB plate with antibiotics. After incubation, the single colony was transferred into LB media (2 ml) with antibiotics, and incubated at 30°C with shaking for one hour. Then the cultures were used to inoculate sterile media (50 ml) in flasks (250 ml) to an initial absorbance A_{600} of 0.01; the cultures were aerated by shaking (150 rpm) in an incubator at 30°C for bioluminoassays *in vivo.* Cell culture density was monitored at 600 nm (A_{600}) with cuvettes (1-cm) in Spectrophotometer Kontron Uvikon 930.

RESULTS AND DISCUSSION

Nucleotide Sequence of the pk1 Gene Encoding Pyruvate Kinase I from P. leiognathi

Plasmid pHC4 carries a ~2.1-kb *Hin*dIII partial digested *P. leiognathi* ATCC 25521 genomic DNA. It contains the *luxZ* gene, which is a regulatory gene enables to enhance bioluminescence of the *lux* operon (16), and others. Partial 3′-end nu-cleotide sequence of the *pkI* gene (GenBank accession No. AF019143) from *P. lei-ognathi* has been determined, and the encoded pyruvate

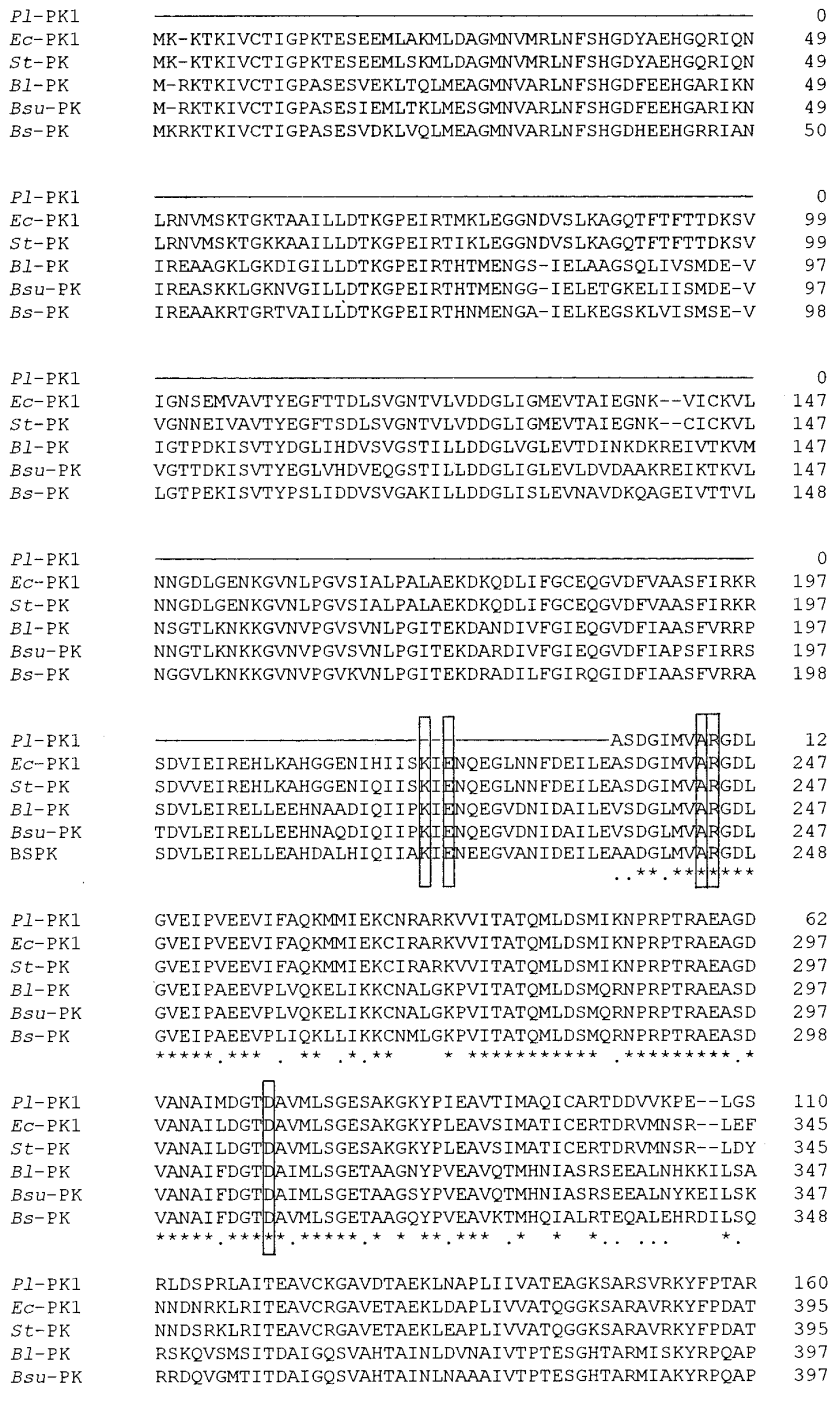

FIG. 2. Alignment and comparison of pyruvate kinase (PKI or PK) amino acid sequences from *P. leiognathi* ATCC 25521 (*Pl*-PKI) and *E. coli* (*Ec*-PKI), *S. typhi-murium* (*St*-PK), *B. licheniformis* (*Bl*-PK), *B. stearothermophilus* (*Bsu*-PK) and *B. subtilis* (*Bs*-PK). Asterisks (*) indicate that the amino acid residues are identical, and dot (.) indicate that the amino acid residues are similar. The key-amino acid residues K220, E222, A243 and R244 are illustrated in box.

kinase I is deduced. Nu-cleotide sequence (780 bp) and open reading frame (ORF from 3rd to 709th bp) of the *pkI* gene is shown in Fig. 1. Pyruvate kinase I encoded by the *pkI* gene is the key enzyme of glycolysis, which converts phosphoenol pyruvate, ADP, H^+ to py-ruvate, ATP. The reaction catalyzed by pyruvate kinase is essentially irreversible reaction that serve as control site for glycolysis. It also is concerned with the 2, 3-diphospho-

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Bs-PK       RTKESQTTITDAIGQSVAHTALNLDVAAIVTPTVSGKTPQMVAKYRPKAP   398
            . .     **.*. .....** .*..  *...* .*.... . ** * *

Pl-PK1      ILAVTTNTKTAAQLCLSKGVTPVVVDSIESTDAFYLRGKELALETGLGAK   210
Ec-PK1      ILALTTNEKTAHQLVLSKGVVPQLVKEITSTDDFYRLGKELALQSGLAHK   445
St-PK       ILALTTNEVTARQLVLSKGVVSQLVKEINSTDDFYRLGKDVALQSGLAQK   445
Bl-PK       IVAVTVNDAVSRKLSLVFGVFATSGQNHSSTDEMLEKAVQKSLDTGIVRH   447
Bsu-PK      IVAVTVNDSISRKLALVSGVFAESGQNASSTDEMLEDAVQKSLNSGIVKH   447
Bs-PK       IIAVTSNEAVSRRLALVWGVYTKEAPHVNTTDEMLDVAVDAAVRSGLVKH   448
            *.*.* *. .. .* *  ** .      ..**..   . . .. .*.  .

Pl-PK1      GDIVVMVSGALVA-SGTTNTASVHVL                          228
Ec-PK1      GDVVVY---------GFWCTGTERHY                          462
St-PK       GDVVVMVSGALVPS-GTTNTASVHVL                          470
Bl-PK       GDLIVITAG-AVGEAGTTNLMKVYVVGDVVAKGQGIGRKSAFGEVVIAQN  496
Bsu-PK      GDLIVITAG-TVGESGTTNLMKVHTVGDIIAKGQGIGRKSAYGPVVVAQN  496
Bs-PK       GDLVVITAGVPVGETGSTNLMKVHVISDLLAKGQGIGRKSAFGKAVVAKT  498
            **..*          *........

Bl-PK       AQEAAKKMKDGAVLVTKSTDRDMMASLEKAAALITEEGGLTSHAAVVGLS  546
Bsu-PK      AKEAEQKMTDGAVLVTKSTDRDMIASLEKASALITEEGGLTSHAAVVGLS  546
Bs-PK       AEEARQKMVDGGILVTVSTDADMMPAIEKAAAIITEEGGLTSHAAVVGLS  548

Bl-PK       LGIPVIVGMENATSILKEGEDITVDSARGAVYKGRASVL   585
Bsu-PK      LGIPVIVGLENATSILTDGQDITVDASRGAVYQGRASVL   585
Bs-PK       LGIPVIVGVENATTLFKDGQEITVDGGFGAVYRGHASVL   587
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FIG. 2—*Continued*

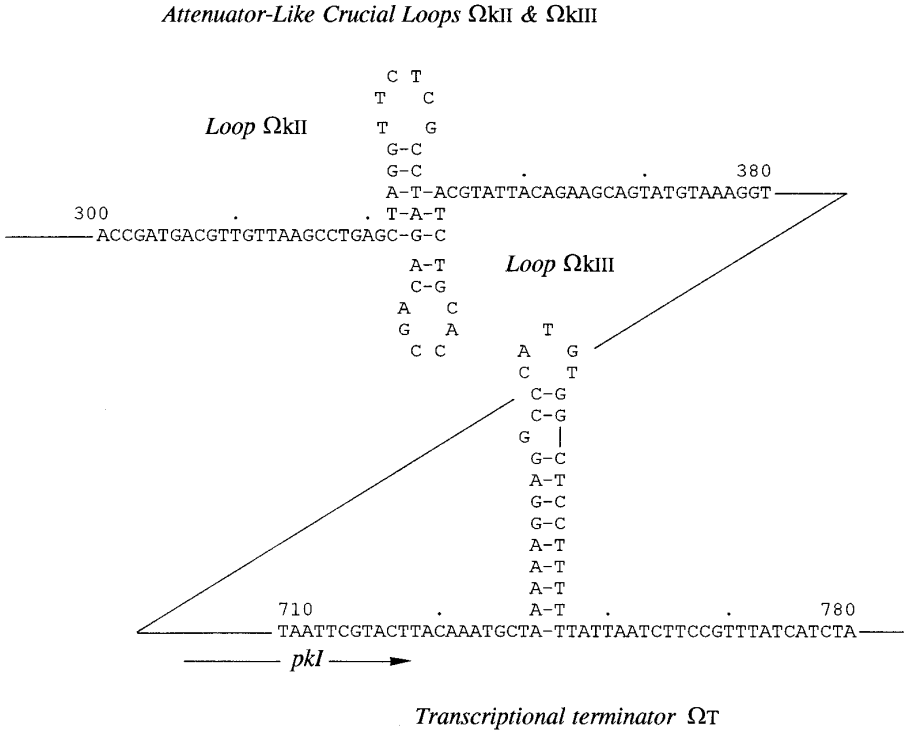

FIG. 3. Secondary structures of attenuator-like crucial loop ΩkII-ΩkIII and trans-criptional terminator ΩT resided in and behind the *pkI* gene of *P. leiognathi*.

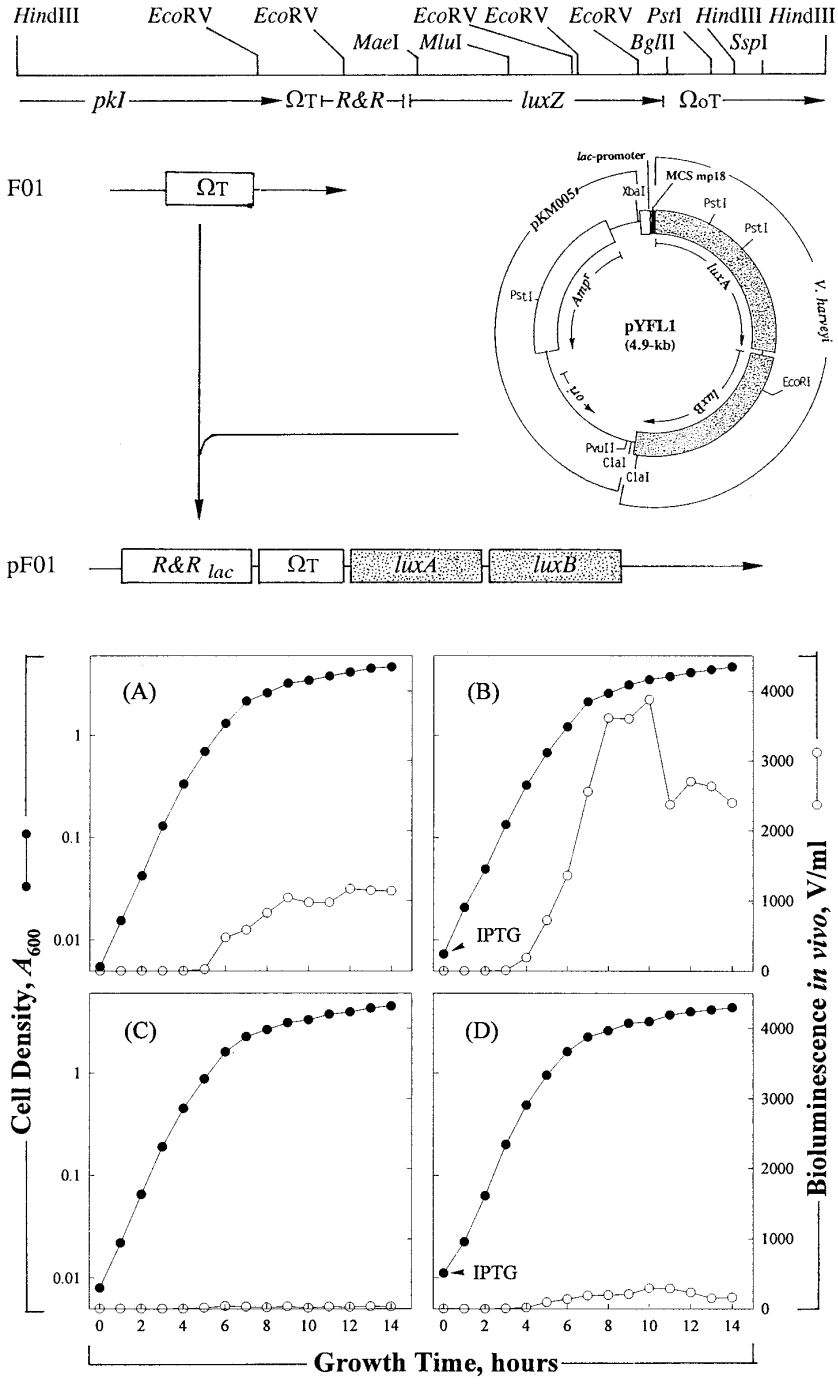

FIG. 4. Construction of pF01 for functional analysis of transcriptional termina-tor ΩT resided behind the *pkI* gene of *P. leiognathi*. The restriction map of pHC4 is shown; whereas the related loci of the *pkI*, *luxZ* genes, ΩT and *R&R* are illustrated. pF01 was constructed by cloning the F01 DNA fragment of loop ΩT into termina-tor-proving vector pYFL1, which carried the *luxA* and *luxB* genes of luciferase as reporter. Growth curve and bioluminescence of plasmid in *E. coli* JM103y are shown; whereas the plasmid indicated as (A) for pYFL1 as control, (B) for pYFL1 with IPTG induction as control, (C) for pF01, and (D) for pF01 with IPTG induc-tion. IPTG (0.2 mM) was added to medium at initiation. Symbols: ●, cell density A_{600}, and ○, bioluminescence *in vivo*.

glycerate (2, 3-DPG) accumulation, which is a regulator of oxygen trans-port. Pyruvate kinase I [ATP:pyruvate 2-O-phosphotransferase, EC 2.7.1.40] en-coded by *E. coli* *pkI* gene has a calculated M_r of ~50 kD and comprises 462 amino acid residues (19). Here shown the C-terminal 345 amino acid residues (~3/5) of pyruvate kinase I en-

coded by the *pkI* gene of *P. leiognathi.* The *pkI* gene of *P. leiognathi* is identified by homology to the *pkI* gene of *E. coli* and *pk* gene of *Sal-monella typhimurium.*

Alignment and Comparison of Pyruvate Kinase Is from Different Species

The specific gene of *P. leiognathi* is confirmed as the *pkI* gene by homology of the encoded protein with pyruvate kinase I of *E. coli* and *S. typhimurium* (19-20). Alignment and comparison of pyruvate kinase Is and pyruvate kinases from these species is shown in Fig. 2; there is 87.2% homologous (71.0% identity and 16.2% similarity); and there is 90.7% homologous (74.8% identity and 15.9% similarity) between these of *P. leiognathi* and *E. coli.* Pyruvate kinase Is from these species are also homologous with pyruvate kinases from *Bacillus subtilis, B. stearothermophi-lus* and *B. licheniformis* (20-24); only the C-terminal of pyruvate kinases from *Baci-lli* are ~100 residues longer than pyruvate kinase Is from *P. leiognathi, E. coli* and *S. typhimurium,* the physiological significance is not really known. 3-D structure re-vealed that pyruvate kinase is folded into four different domains; residues ~1-40 form a small domain involved in subunit contacts in the tetrameric molecule; residues ~40-115 and ~220-380 form an α/β-barrel domain that provides the active center catalytic key-amino acid residues for substrate binding; residues ~115-220 loop out to fold an antiparallel β sheet; residues ~380-460 form an open twisted α/β domain (21). The key-amino acid residues are conserved in pyruvate kinases and pyruvate kinase Is, *i.e.* K220 is concerned with enzyme activity; E222, A243 and R244 resi-dues are related to Mg^{+2}-binding (22). It elucidates that pyruvate kinases and pyru-vate kinase Is, the key enzyme of glycolysis and essential for life, are conserved for the structure and function during evolution.

Functional Analysis of Transcriptional Terminator WT Lay Behind the pkI Gene

Nucleotide sequence analysis elucidates that potential hairpin loops ΩkI (65th to 72nd bp; $\Delta G^o = -4.6$ kcal/mol), ΩkII (324th to 339th bp; $\Delta G^o = -7.2$ kcal/mol), ΩkIII (335th to 354th bp; $\Delta G^o = -4.4$ kcal/mol) could be formed inside the *pkI* gene (as shown in Fig. 1). There loops ΩkII, ΩkIII overlapped and formed attenuator-like crucial loops (as shown in Fig. 3) might be concerned with the sub-regulation of the gene. In addition, the potential hairpin loop ΩT (729th to 756th bp; $\Delta G^o = -10.4$ kcal/mol), which followed by a run of U residues, could be formed behind the *pkI* gene and before the regulatory region *R&R″* of the *luxZ* and related genes appears a potential transcriptional terminator. Terminator-proving vector pYFL1 was used to do functional analysis for the putative transcriptional terminator ΩT. The F01 248-bp (638th to 885th bp) *Eco*RV DNA fragment included the potential hairpin loop ΩT was cloned into pYFL1 to construct pF01. Bioluminoassays *in vivo* elucidates that even with IPTG induction, loop ΩT could be formed to terminate the transcrip-tion and repress gene expression (shown in Fig. 4); it confirms that loop ΩT indeed functions as transcriptional terminator for the *pkI* and related gene.

Gene Order of the pkI Gene with the luxZ Gene

Nucleotide sequence analysis reveals that the *pkI* gene is simply linked to the *luxZ* gene, and run in the same direction; the gene order is *-pkI-ter-→R&R"-luxZ-ter"→* (*R&R:* regulatory region; *ter,* transcriptional terminator). As depicted, *ter* is transcriptional terminator for the *pkI* and related genes, and *R&R″* is the regulatory region and *ter″* is transcriptional terminator for the *luxZ* gene (16). The intrinsic se-quence 320-bp lay between these genes, which included the transcriptional termina-tor *ter* of the *pkI* gene and the regulatory region *R&R″* of the *luxZ* gene, divides the genes to two operons in genome; it infers that the *pkI* and related genes are simply linked to the *luxZ* gene in *P. leiognathi.* However, pyruvate kinase is the key enzy-me for final step of glycolysis concerned with glucose metabolism and 2, 3-DPG accumulation, and related to cAMP formation and O_2 transport. As known, O_2 and cAMP-CRP involved in bioluminescence reaction and regulation of the *lux* operon, it implies that *pkI* gene closely linked to the *luxZ* gene might be significant for bio-luminescence in *P. leiognathi,* but it is not really known.

ACKNOWLEDGMENTS

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